Journal of Molecular and Cellular Cardiology 47 (2009) 391–399

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Chronic hypoxia inhibits MMP-2 activation and cellular invasion in human cardiac myofibroblasts

Kirsten Riches [a,b,1], Michael E. Morley [a,b,1], Neil A. Turner [a,b], David J. O'Regan [a,c], Stephen G. Ball [a,b], Chris Peers [a,b], Karen E. Porter [a,b,*]

[a] Multidisciplinary Cardiovascular Research Centre (MCRC), University of Leeds, Leeds LS2 9JT, UK
[b] Division of Cardiovascular and Neuronal Remodelling, Leeds Institute of Genetics, Health and Therapeutics (LIGHT), University of Leeds, Leeds LS2 9JT, UK
[c] Department of Cardiac Surgery, the Yorkshire Heart Centre, Leeds General Infirmary, Leeds LS1 3EX, UK

ARTICLE INFO

Article history:
Received 21 April 2009
Received in revised form 21 May 2009
Accepted 2 June 2009
Available online 11 June 2009

Keywords:
Collagen
MMP-2
Hypoxia
Invasion
Migration
MT1-MMP

ABSTRACT

Cardiac myofibroblasts are pivotal to adaptive remodelling after myocardial infarction (MI). These normally quiescent cells invade and proliferate as a wound healing response, facilitated by activation of matrix metalloproteinases, particularly MMP-2. Following MI these reparative events occur under chronically hypoxic conditions yet the mechanisms by which hypoxia might modulate MMP-2 activation and cardiac myofibroblast invasion have not been investigated. Human cardiac myofibroblasts cultured in collagen-supplemented medium were exposed to normoxia (20% O_2) or hypoxia (1% O_2) for up to 48 h. Secreted levels of total and active MMP-2 were quantified using gelatin zymography, TIMP-2 and membrane-associated MT1-MMP were quantified with ELISA, whole cell MT1-MMP by immunoblotting and immunocytochemistry and MT1-MMP mRNA with real-time RT-PCR. Cellular invasion was assessed in modified Boyden chambers and migration by scratch wound assay.

In the human cardiac myofibroblast, MT1-MMP was central to MMP-2 activation and activated MMP-2 necessary for invasion, confirmed by gene silencing. MMP-2 activation was substantially attenuated by hypoxia ($P<0.001$), paralleled by inhibition of myofibroblast invasion ($P<0.05$). In contrast, migration was independent of either MT1-MMP or MMP-2. Reduced membrane expression of MT1-MMP ($P<0.05$) was responsible for the hypoxic reduction of MMP-2 activation, with no change in either total MMP-2 or TIMP-2. In conclusion, hypoxia reduces MMP-2 activation and subsequent invasion of human cardiac myofibroblasts by reducing membrane expression of MT1-MMP and may delay healing after MI. Regulation of these MMPs remains an attractive target for therapeutic intervention.

1. Introduction

Fibroblasts account for up to two-thirds of the total cell number in the normal human heart [1] and maintain the integrity of myocardial structure through controlled proliferation and extracellular matrix (ECM) turnover [2,3]. Adverse remodelling following myocardial infarction (MI) is characterised by myocyte death, hypertrophy of surviving myocytes, proliferation, migration and invasion of cardiac myofibroblasts and fibrosis [3–5]. An important part of this process is the transformation of normally quiescent cardiac fibroblasts into the proliferative and invasive myofibroblast phenotype that can alter the structure of the cardiac interstitium through increased expression and activation of matrix-degrading metalloproteinases (MMPs), particularly the gelatinases MMP-2 and MMP-9 [6,7]. Gelatinase activation is a prerequisite for both proliferation and invasion *in vivo* and is required to promote myocardial infarct healing and adaptive remodelling. However, in the long-term this can lead to adverse myocardial remodelling characterised by impaired contractile function and fibrosis.

Unlike MMP-9, which is primarily regulated at the transcriptional level, MMP-2 is constitutively expressed by cardiac myofibroblasts and secreted in a latent form (proMMP-2). Activation of proMMP-2 takes place at the cell membrane and requires the participation of both membrane-type 1 matrix metalloproteinase (MT1-MMP) and tissue inhibitor of metalloproteinases-2 (TIMP-2) in cancer cells [8]. MT1-MMP is itself expressed in a latent form and processed intracellularly to its active species by furin, a proprotein convertase that cleaves precursor proteins to their biologically active state [9]. Generation of active MT1-MMP and its transfer to the cell membrane is a critical step in MMP-2 activation [8] and, together with TIMP-2, facilitates membrane binding of proMMP-2 to MT1-MMP. Although the main role of TIMP-2 is that of physiological inhibition of MMP-2

* Corresponding author. Division of Cardiovascular and Neuronal Remodelling, Leeds Institute of Genetics, Health and Therapeutics (LIGHT), University of Leeds, Leeds LS2 9JT, UK. Tel.: +44 113 3434806; fax: +44 113 3434803.
E-mail address: medkep@leeds.ac.uk (K.E. Porter)
[1] These authors contributed equally to the manuscript.

0022-2828/
doi:10.1016/j.yjmcc.2009.06.002

and MT1-MMP by post-translational regulation [10], it is also essential for cell membrane binding of proMMP-2. This binding in turn promotes activation of proMMP-2 which facilitates ECM degradation [11].

Acute coronary events such as MI lead to profound myocardial injury and are inevitably associated with periods of inadequate oxygen supply (hypoxia) to the myocardium. Hypoxia itself is a major cue for tissue remodelling in the cardiovascular system, in part via MMP activation [12,13]. Indeed, distinct responses in different cell types in a variety of species have been shown to be induced as a result of hypoxic insult. Typically, hypoxia has been reported to increase cellular activation of MMP-2 as demonstrated in monkey endothelial cells [14], rat cardiac fibroblasts [15] and human HT-1080 fibrosarcoma cells [16]. Interestingly however, although chronic hypoxia increased MMP-2 secretion in human endothelial cells, a decrease in its activation was observed [17]. Similarly, an inhibitory effect of chronic hypoxia on MMP-2 activation has been documented in human endometrial stromal cells [17,18] and proximal tubular cells [19]. It is clear therefore, that hypoxia-induced effects on MMP-2 activation are both species and cell-type specific and, importantly, the underlying mechanisms are not consistent. Since any changes to the regulation of matrix deposition and degradation in the myocardium have the potential to create deleterious effects on myocardial function, the present study was designed to investigate specifically the influence of chronic hypoxia on collagen-induced MMP-2 activation and invasion of human cardiac myofibroblasts.

2. Materials and methods

2.1. Reagents

All cell culture reagents were purchased from Invitrogen (Paisley, UK), except foetal calf serum (FCS) that was from Biosera (Ringmer, East Sussex, UK). Invasion and migration assay chambers and inserts were from BD Biosciences (Oxford, UK). Real-time PCR reagents were from Applied Biosystems (www.appliedbiosytems.com).

2.2. Cell culture

Biopsies of human atrial appendage were obtained from patients undergoing elective coronary artery bypass grafting (CABG). Ethical committee approval and informed patient consent were obtained and the investigation conformed to the principles outlined in the Declaration of Helsinki. Primary cultures of atrial cardiac myofibroblasts were harvested and cultured in full growth medium (Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin) essentially as we have described previously [20]. Cells were characterised as myofibroblasts by co-expression of α-smooth muscle actin (α-SMA) and vimentin [21,22]. Experiments were performed on early passage cells (2–5) from different patients.

HT-1080 cells were cultured in fibroblast full growth medium (DMEM plus 10% FCS) and serially passaged by trypsinisation.

2.3. Collagen supplemented myofibroblast culture

Cardiac myofibroblasts (3×10^5) were plated in full growth medium and incubated overnight. Medium was then exchanged for serum free medium (SFM) for 48 h. Serum-starved cells were transferred to fresh medium supplemented with 0.5% FCS and 50 μg/ml Type I collagen (Calbiochem) and transferred to humidified incubators at 37 °C for 24–48 h in normoxia (5% CO_2 in air) or hypoxia (1% O_2, 5% CO_2, 94% N_2). Type I collagen culture conditions were used to represent the *in vivo* environment of cardiac myofibroblasts. Samples of conditioned media (CM) were collected for zymography and/or immunoassay (ELISA).

2.4. Gelatin zymography

Gelatin zymography and subsequent densitometric analysis were performed on myofibroblast CM as we have described previously [23,24]. Optimised loading volumes were determined by serial dilution of CM to ensure that band intensity was proportional to MMP-2 zymographic activity. CM from HT-1080 cells (a human fibrosarcoma cell line that constitutively secretes MMP-2) [16] was included on each gel as a positive control.

2.5. ELISA

MMP-2 or TIMP-2 protein levels in CM were quantified by ELISA (R&D Systems, Abingdon, UK). Membrane-bound MT1-MMP was measured by ELISA (Amersham Biosciences, Amersham, UK) of membrane fractions prepared following 15 min exposure of cells to 2 ml extraction buffer (50 mM Tris–HCl, pH 7.6; 1.5 mM NaCl; 0.5 mM $CaCl_2$; 1 μM $ZnCl_2$; 0.01% Brij-35; 0.25% Triton X-100) at 4 °C.

2.6. Real-time RT-PCR

Myofibroblasts were cultured in collagen-supplemented medium in normoxia/hypoxia for 24–48 h. RNA extraction and reverse transcription were performed as we described previously [25]. Real-time PCR with human MT1-MMP (Hs00237119_m1) primers and Taqman probes was performed using the Applied Biosystems 7500 Real-Time PCR System. Data are expressed as a percentage of β-actin mRNA levels (Hs99999903_m1 primers) using the formula $2^{-\Delta CT}\times100$.

2.7. Whole cell MT1-MMP levels

MT1-MMP levels within the whole cell were analysed by immunocytochemistry and immunoblotting. For fluorescence immunocytochemistry, cells were fixed in 4% paraformaldehyde and stained using a previously described protocol [26]. For immunoblotting, non-reduced cell lysates were prepared by scraping into extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 0.05% Brij-35), sonicating 6×10 s (4 °C) and centrifugation for 15 min (8000 ×*g*, 4 °C) to pellet cellular debris. Lysates were subsequently electrophoresed on a 7.5% SDS-PAGE gel, transferred to PVDF membrane and visualised using MT1-MMP antibody (ab38971; AbCam, Cambridge, UK) and enhanced chemiluminescence.

2.8. Inhibition of lysosomal degradation

Cells were cultured in normoxia/hypoxia in 24-well plates in collagen-supplemented media with a range of concentrations of the lysosomal inhibitor bafilomycin-A1 (Sigma-Aldrich, Poole UK). Following 48 h incubation, CM was collected and MMP-2 activation analysed by gelatin zymography.

2.9. Cardiac myofibroblast phenotype

Cells cultured for 48 h in normoxia/hypoxia in 8-well chamber slides, were fixed and stained with antibodies to α-SMA and vimentin, firmly establishing their myofibroblast phenotype in these conditions without additional transformation, as previously described [22].

2.10. Proliferation assays

Proliferation assays were performed as we described previously [22]. Cardiac myofibroblasts were cultured in parallel in normoxia and hypoxia in medium containing 0.5% and 10% FCS. Cell numbers were monitored over a 7-day period.

2.11. Invasion assays

Invasion assays were performed essentially as we described previously [24] using a modified Boyden chamber technique with Matrigel basement membrane matrix coated membranes (BD Biosciences, Oxford, UK). Chemoattractant (2.5% FCS in DMEM) was loaded into the lower chamber and cardiac myofibroblasts were cast in Type I collagen gels in the upper chamber (1×10^5 cells) in SFM supplemented with 0.25% bovine serum albumin (BSA). After a 48-h incubation period in normoxic or hypoxic conditions, processing and evaluation were performed [24]. For gene silencing studies, transfected myofibroblasts were incubated for 24 h in media supplemented with 0.4% FCS prior to the assay. After 22 h, cell supernatants were collected from the upper chambers for analysis by gelatin zymography and invasion quantified by counting the invaded cells in ten random high power fields (×400) for each membrane [24].

2.12. Wound scratch migration assays

Wound scratch migration assays were performed essentially as described previously [27] with minor modifications. A single linear wound was made with a 10 ml pipette tip in confluent cultures of 48 h serum-starved cardiac myofibroblasts, then washed gently with PBS to remove cellular debris. The cells were transferred to fresh DMEM supplemented with 0.5% FCS and 50 μg/ml Type I collagen, and incubated at 37 °C under normoxic and hypoxic conditions. After 48 h cells were photographed using phase contrast microscopy (×40 magnification). The number of cells migrating beyond a marker 500 μm from the wound edge was counted.

2.13. Transfection of siRNA oligonucleotides

Cardiac myofibroblasts (3×10^5) were incubated overnight in medium supplemented with 0.4% FCS, prior to transfection with siRNA targeting MMP-2 (5′-CGGACAAAGAGUUGGCAGU-3′, 200 nM, Eurogentec) or MT1-MMP (5′-GGAAUGAGGAUCUGAAUGG-3′, 50 nM, Ambion) using Lipofectamine 2000 (Invitrogen), or mock transfected (Lipofectamine without siRNA) as we have previously described [25,28]. Gelatin zymography and ELISA were performed to confirm efficient silencing of MMP-2 and MT1-MMP respectively.

2.14. Statistical analysis

All results are expressed as mean ± SEM with n representing the number of experiments on cells from different patients. Differences between treatment groups were analysed using paired ratio t-tests or one-way repeated measures ANOVA with a Bonferroni post-hoc test (GraphPad Prism software, www.graphpad.com). $P<0.05$ was considered statistically significant.

3. Results

3.1. Type I collagen induces MMP-2 activation that is attenuated by chronic hypoxia

Myofibroblasts cultured in the presence of Type I soluble collagen under normoxic conditions showed significant activation of MMP-2 compared with cells cultured in the absence of collagen (Fig. 1A), as evidenced by the appearance of a lower molecular weight zymogen band (62 kDa) in addition to the 72 kDa proprotein. There were no differences in secreted levels of total MMP-2 between cells cultured in the absence or presence of collagen (data not shown). After exposure to chronic hypoxia (1% O_2, 48 h) collagen-induced MMP-2 activation was significantly and consistently attenuated by ~50% (Figs. 1A, B). This was not the result of cell death in hypoxia, since cell numbers were similar over 7 days (Fig. 4F), confirming that differences in MMP-2 activation were not attributable to variations in cell number. We repeated these experiments using the human fibrosarcoma cell line HT-1080 in which hypoxic regulation of MMP-2 secretion and activation has previously been characterised [16]. In marked contrast to the myofibroblasts, but in agreement with this previous report, HT-1080 cells exhibited a significant increase in MMP-2 activation

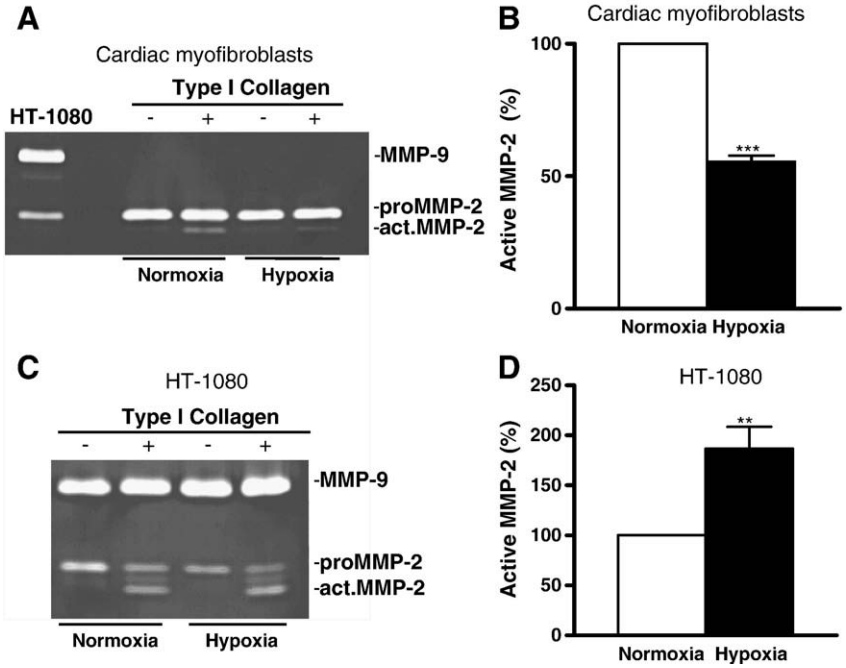

Fig. 1. Effect of collagen and hypoxia on MMP-2 activation in cardiac myofibroblasts and HT-1080 cells. (A) Representative zymography of conditioned media collected from myofibroblasts cultured without (−) or with (+) 50 μg/ml collagen in normoxia (20% O_2) or hypoxia (1% O_2) for 48 h. (B) Densitometric quantification of active MMP-2. Data are expressed relative to cells cultured in collagen and normoxia. ***$P<0.001$ ($n=6$). (C) Representative zymogram of HT-1080 conditioned media under the same experimental conditions (note two bands showing cleavage products). (D) Densitometric quantification of combined intermediate and fully active MMP-2 **$P<0.01$ ($n=5$).

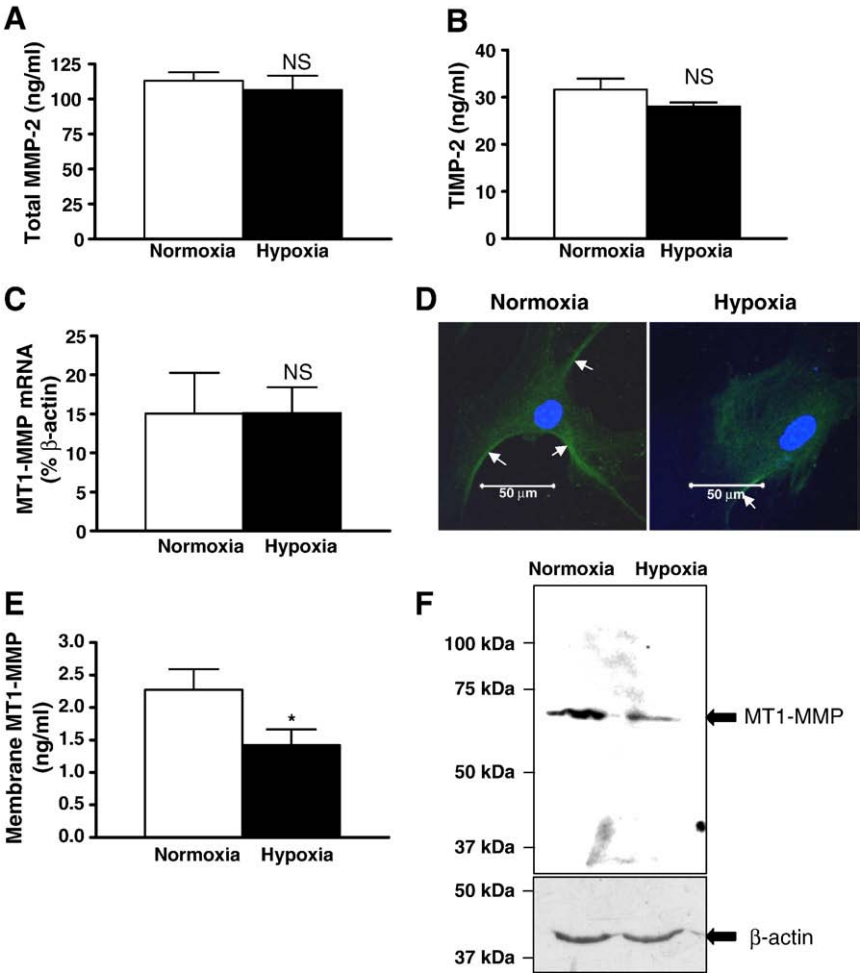

Fig. 2. Effect of hypoxia on total MMP-2, TIMP-2 and MT1-MMP expression. Cardiac myofibroblasts were cultured with collagen under normoxia or hypoxia for 48 h. Conditioned media were analysed by ELISA for (A) total (pro- and active) MMP-2 or (B) total TIMP-2. NS = not significant ($n = 5$). (C) Cells were cultured with collagen under normoxia or hypoxia for 24 h before extracting RNA and performing real-time RT-PCR. Data shows quantification of MT1-MMP mRNA levels as a percentage of β-actin levels. NS = not significant ($n = 5$). (D) Immunofluorescent detection of MT1-MMP localisation (green) following 48 h of collagen-supplemented culture under normoxic or hypoxic conditions. Nuclei stained with DAPI (blue). Scale bar = 50 μm. (E) Membrane expression of MT1-MMP (ELISA) after 48 h normoxia or hypoxia. *$P < 0.05$ ($n = 5$). (F) Representative immunoblot of whole cell MT1-MMP expression in lysates prepared following 48 h of collagen-supplemented culture in normoxia and hypoxia with β-actin as loading control.

(~200%) compared with normoxic controls (Figs. 1C, D). In further agreement with this same report, an intermediate band of MMP-2 activation (~68 kDa) was observed in HT-1080 media (Fig. 1C). Thus, the effects of hypoxia observed in human cardiac myofibroblasts were markedly different from those seen in HT-1080 cells.

3.2. Hypoxia attenuates MT1-MMP but does not affect TIMP-2 or MMP-2

Activation of MMP-2 at the cell membrane requires formation of a ternary complex of MT1-MMP, TIMP-2 and proMMP-2 [8]. We therefore investigated whether the observed reduction in MMP-2 activation in myofibroblasts cultured in hypoxia might be due to changes in total MMP-2, TIMP-2 and/or MT1-MMP expression. Densitometric analysis of gelatin zymograms demonstrated no change in total MMP-2 secretion in response to hypoxia (Fig. 1A), and this was confirmed by ELISA (Fig. 2A). Similarly, hypoxia had no effect on TIMP-2 protein levels (Fig. 2B). We next quantified levels of MT1-MMP mRNA and, because it is the active form of MT1-MMP that traffics to the plasma membrane to facilitate MMP-2 activation [11,29], we also measured MT1-MMP protein in cell membrane and whole cell fractions using ELISA and immunoblotting. Hypoxia did not alter steady-state MT1-MMP mRNA levels (Fig. 2C). Immunocytochemistry revealed a general diffuse staining within the cytoplasm, with a more concentrated localisation of MT1-MMP at the cell periphery in normoxia. This association with the membrane was not apparent when cells were cultured under chronically hypoxic conditions (Fig. 2D). In keeping with the immunofluorescence findings, measurement of MT1-MMP in cell membrane preparations using ELISA showed a ~40% reduction under hypoxic conditions (Fig. 2E). Measurement of total MT1-MMP in whole cell lysates (ELISA and immunoblotting) also indicated a ~40% decrease with hypoxia (Fig. 2F).

3.3. Lysosomal degradation of MT1-MMP is not enhanced by hypoxia

As hypoxia reduced MT1-MMP protein without affecting MT1-MMP mRNA, we looked for evidence of hypoxia-induced MT1-MMP degradation. MT1-MMP is degraded by lysosomes [30], therefore cells were cultured with increasing concentrations of the lysosomal inhibitor bafilomycin-A1 to determine whether the decrease in MMP-2 activation could be attributable to enhanced degradation of MT1-MMP. Zymographic analysis of CM from cells cultured in normoxia revealed that bafilomycin-A1 increased activation of MMP-2 in a concentration-dependent manner (Figs. 3A, B). However, irrespective of bafilomycin-A1 treatment, cells cultured under hypoxic conditions exhibited attenuation of MMP-2 activation compared with normoxic counterparts (Figs. 3A, B).

3.4. Hypoxia inhibits cardiac myofibroblast invasion, but not migration

Cellular invasion requires two distinct phases; degradation of ECM followed by cellular migration towards a chemotactic stimulus. A 48 h hypoxic period led to a significant inhibition of myofibroblast invasion of almost 50% compared with normoxic cells (Figs. 4A, B). This was associated with a corresponding decrease in MMP-2 activation in the supernatants from cells used in these experiments (Figs. 1A, B). However, in the wound scratch assay, despite also observing a consistent inhibition of MMP-2 activation, there was no difference in cellular migration between the normoxic and hypoxic treatment groups (Figs. 4C, D). Immunocytochemical analysis of normoxic and hypoxic cells for the markers α-SMA and vimentin confirmed that the cells had retained their myofibroblast phenotype (Fig. 4E). These results together strongly suggest that MMP-2 activation is a prerequisite for cardiac myofibroblast invasion, but not migration.

3.5. MT1-MMP is required for collagen-stimulated MMP-2 activation and invasion, but not migration

Since we had demonstrated that hypoxia reduced invasion (but not migration), and also reduced both MT1-MMP protein levels together with MMP-2 activity, we sought to establish whether these effects were sufficient to inhibit myofibroblast invasion. Myofibroblasts transfected with siRNA targeting MT1-MMP or MMP-2 were cultured in collagen for 48 h and supernatants subjected to zymography. Total MMP-2 was selectively reduced by ~50% with MMP-2 gene silencing, but was unaffected by MT1-MMP siRNA (Figs. 5A, B). Cells transfected with MT1-MMP siRNA exhibited a ~50% decrease in membrane MT1-MMP expression (Fig. 5C). Importantly, selective silencing of either MT1-MMP or MMP-2 resulted in a significant reduction in active MMP-2 levels (Fig. 5D), to a level comparable with that observed in myofibroblasts during hypoxic culture.

The functional roles of both MT1-MMP and MMP-2 were examined by studying siRNA-treated cells in invasion and migration assays. Silencing of either MT1-MMP or MMP-2 resulted in decreased cellular invasion of ~50% (Fig. 5E). Parallel wound scratch assays revealed no difference in myofibroblast migration between control and transfected groups, confirming that cell motility *per se* is independent of MT1-MMP and MMP-2 activity (Fig. 5F). These observations concur with our observed effects of hypoxia on cell function, thus confirming the pivotal role of membrane-associated MT1-MMP activity in the mechanism of MMP-2 activation and invasion in this cell type.

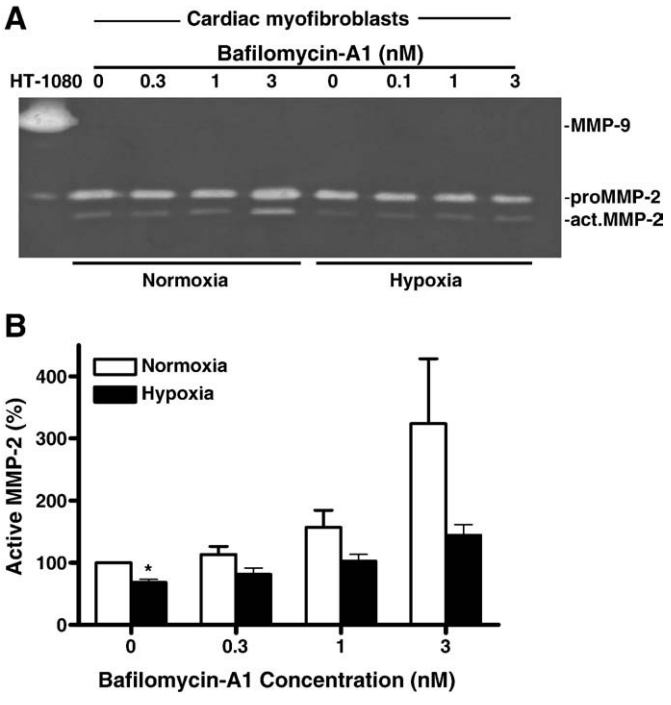

Fig. 3. Effect of lysosomal inhibition on MMP-2 activation. Cells were cultured for 48 h in normoxia and hypoxia with a range of concentrations of the lysosomal inhibitor bafilomycin-A1. (A) Representative zymography of CM collected from myofibroblasts cultured with 50 μg/ml collagen and 0.3–3 nM bafilomycin-A1 in normoxia and hypoxia for 48 h. (B) Densitometric quantification of active MMP-2. Data are expressed relative to cells cultured in vehicle and normoxia. *$P<0.05$ for the effect of hypoxia in the absence of bafilomycin-A1 ($n=5$). ANOVA values: $P=0.0263$ (normoxia), $P=0.0001$ (hypoxia).

4. Discussion

This study reveals mechanisms unique to human cardiac myofibroblasts of potential clinical significance, underscoring the importance of selecting appropriate experimental models. Cardiac myofibroblasts reside in a fibrillar collagen matrix *in vivo*, and we demonstrate here that collagen-supplemented *in vitro* cultures provide an appropriate physiological stimulus for activation of MMP-2 in human cardiac myofibroblasts. Moreover, myofibroblasts in culture are phenotypically comparable with their *in vivo* counterparts [29] and similar studies have also been reported for other fibroblast cultures [31]. We show here that MMP-2 activation is a prerequisite for cellular invasion, a process fundamental to promotion of the early, adaptive healing response after myocardial injury and is attenuated by hypoxia.

Considerable evidence supports a role for MMP-2 and MT1-MMP in cellular invasion. ProMMP-2 displays a unique cell surface activation mechanism involving the formation of a trimolecular complex comprising MT1-MMP, TIMP-2 and proMMP-2 [8–10]. Therefore, MMP-2 activation can be regulated by changes in expression and/or activation of any of these molecules. In marked contrast to previously published studies using a variety of cell types [14–17,32], including rat cardiac fibroblasts [15], exposure to chronic hypoxia significantly attenuated MMP-2 activation in human cardiac myofibroblasts. These observations cannot be attributed to methodological variance since, in agreement with the study of Fahling et al. [16], we also observed a significant increase in activation of MMP-2 by hypoxia in HT-1080 cells. Our findings for human cardiac myofibroblasts therefore represent a novel response to hypoxia.

In vitro studies are usually conducted in an atmosphere of 95% air. This represents an O_2 level of 20%; far greater than any cellular exposure *in vivo*. Our data emphasise the powerful effects of reducing O_2 levels to those observed *in vivo* during clinically important insults such as MI. Clearly, this level of hypoxia has profound effects on protein expression which can be mediated by numerous transcription factors including HIF-1, NFκB and CREB [33].

TIMP-2, in addition to its principal role as the naturally-occurring physiological inhibitor of MMP-2, is also required for its activation, albeit at lower concentrations [34]. In our present study, although TIMP-2 was consistently detected, its concentration in conditioned media of both normoxic and hypoxic myofibroblasts was similar and therefore changes in TIMP-2 levels could not account for our observed changes in MMP-2 activation. It is well established that MT1-MMP is pivotal to MMP-2 activation; however reports are inconsistent with respect to its modulation under hypoxic conditions. That MT1-MMP mRNA was unchanged by hypoxia in our study is in agreement with previous reports [16,32] although both those studies used inherently invasive cell lines and moreover, both reported increased MMP-2 activation in contrast to our observed decrease. However, other studies have reported either increased [35] or decreased [17] MT1-MMP mRNA levels, further emphasising the species and cell-type specific response to hypoxia. Here, although MT1-MMP mRNA levels were not modulated by hypoxia, total levels of protein within the cell

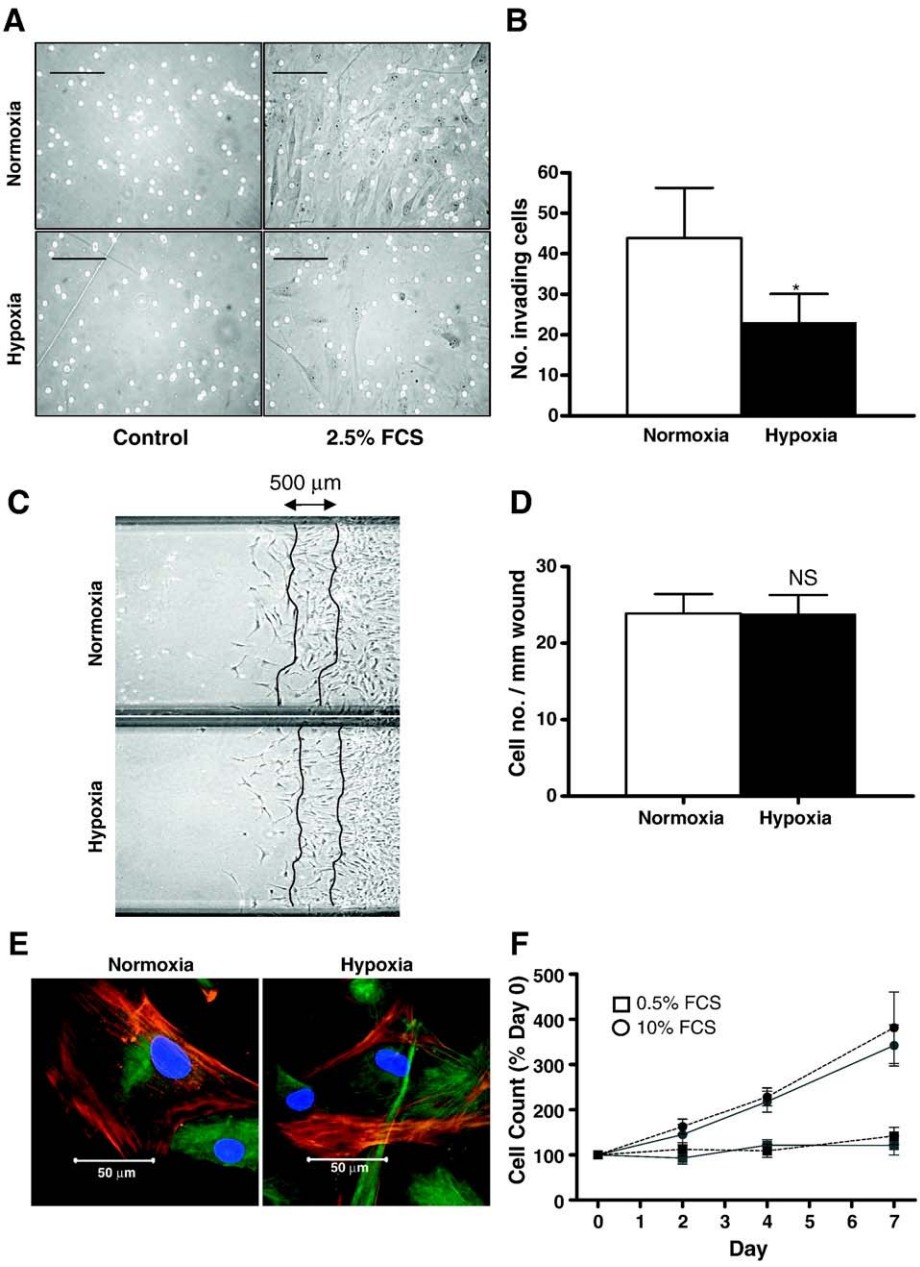

Fig. 4. Effect of hypoxia on cardiac myofibroblast invasion, migration and proliferation. Invasion assays were performed over a 48 h period in normoxia or hypoxia. Membranes were fixed, stained and cell invasion quantified by counting 10 high power (×400) fields per membrane. (A) Representative fields in normoxic and hypoxic culture. Scale bar = 100 μm. (B) Quantification of invaded cells. Data expressed as average cell number per field. *$P<0.05$ ($n=7$). (C) For migration studies, cells were grown to confluence before creating a uniform scratch wound and incubating in collagen and normoxia/hypoxia for a further 48 h. Representative image of wound assay after 48 h, shows cell migration into denuded wound area in normoxia and hypoxia (original magnification ×40). (D). Quantification of migrated cells. Data expressed as number of cells migrated to or further than 500 μm per mm wound width. NS = not significant ($n=5$). (E). Co-localisation of α-SMA (red) and vimentin (green) following 48 h of culture as seen by immunofluorescence microscopy. Nuclei stained with DAPI (blue). Scale bar = 50 μm. (F). 7-day proliferation profile of cells cultured in normoxia (solid line) and hypoxia (hashed line) in 0.5% and 10% FCS.

and most importantly at the cell membrane were attenuated by ~40%. Hypoxia was reported to reduce membrane-associated MT1-MMP in isolated porcine ventricular myocytes [36], but was increased in a breast cancer cell line [32], emphasising a role of MT1-MMP as a key regulator of cellular invasion.

In direct contrast to previous reports in other cell types, chronic hypoxia significantly attenuated myofibroblast invasion in the present study. The reduced availability of membrane-associated MT1-MMP would be anticipated to prohibit ternary complex formation and thus lessen MMP-2 activation, accounting for the significant inhibition of invasion. We therefore demonstrated that transfection of myofibroblasts with MMP-2 siRNA reduced total MMP-2 secretion; additionally transfection with MT1-MMP siRNA resulted in both reduced membrane expression of MT1-MMP and reduced MMP-2 activation. Together these observations clearly delineate an essential role for both molecules in the mechanism of MMP-2 activation in human cardiac myofibroblasts. We therefore performed mechanistic and functional studies in parallel and confirmed that cardiac myofibroblast invasion was unequivocally dependent on MT1-MMP. These findings are in agreement with reports in other fibroblastic cell types [37–40]. Our study also indicated that knockdown of MMP-2 inhibited invasion as in human mesenchymal stem cells [38]. Conversely, others have reported that MMP-2 siRNA did not attenuate invasion by HT-1080 cells [39].

In contrast to the significant effect on myofibroblast invasion, our data indicated that chemotaxis (migration) can proceed independently of changes in oxygen tension. Despite the lack of effect of hypoxia on myofibroblast migration, we did however observe a consistent attenuation of active MMP-2 in fibroblast-conditioned medium from these assays. Similarly, using MMP-2 siRNA or MT1-MMP siRNA-transfected cells, migration was not different from that of mock-transfected cells. Our results concur with a study reporting that migration of dermal fibroblasts from MT1-MMP null mice was comparable with those of wild-type mice [39]. However, again cell type differences are reported with MT1-MMP knockdown decreasing migration in HT-1080 cells [40].

In the present study, membrane expression of active MT1-MMP in human cardiac myofibroblasts was pivotal for hypoxic modulation of both MMP-2 activation and cellular invasion. Hypoxia did not affect MT1-MMP gene expression and hence must modulate its post-transcriptional regulation. Potential targets for hypoxic regulation of MT1-MMP are many, and include activation of the proenzyme by proprotein convertases [41], membrane localisation, shedding, degradation and MT1-MMP phosphorylation [42,43]. The activity of furin, the proprotein convertase responsible for MT1-MMP activation, was increased under hypoxic conditions and therefore could not be accountable for the decreased levels of MT1-MMP (data not shown). Similarly the amount of soluble ("shed") MT1-MMP in myofibroblast-conditioned media was not enhanced by chronic hypoxia (data not shown). Lysosomal inhibition of MT1-MMP degradation with bafilomycin-A1 led to a concentration-dependent increase in MMP-2 activation, as expected. However, this augmentation was observed under both normoxic and hypoxic conditions, thereby ruling out increased lysosomal activity as the mediator of hypoxia-induced decrease in membrane-associated MT1-MMP. Taken together our data suggest that impairment of MT1-MMP protein translation is the most likely target of hypoxia, thereby reducing its availability for ternary complex formation, and consequent inhibition of MMP-2 activation.

Hypoxia is only one of numerous cues that influence cardiac matrix remodelling through effects on MMPs, and although our current study investigated hypoxia *per se*, ischaemia-reperfusion injury is another key regulator of MMP activity that modulates its effects through alternative mechanism(s) [44]. Inflammatory mediators released early after MI also play key roles. For example, TNFα modulates long-term remodelling and ventricular dysfunction via increased MMP-2 activity [45]. In contrast, TGFβ may only play a short-term role; murine studies have reported that anti-TGFβ therapy prior to experimental MI decreased collagen I and MMP-9 mRNA expression after 3 days, but was ineffective by 8 weeks [46]. Activation of MMP-2 post-MI can have positive or negative effects that appear to be species

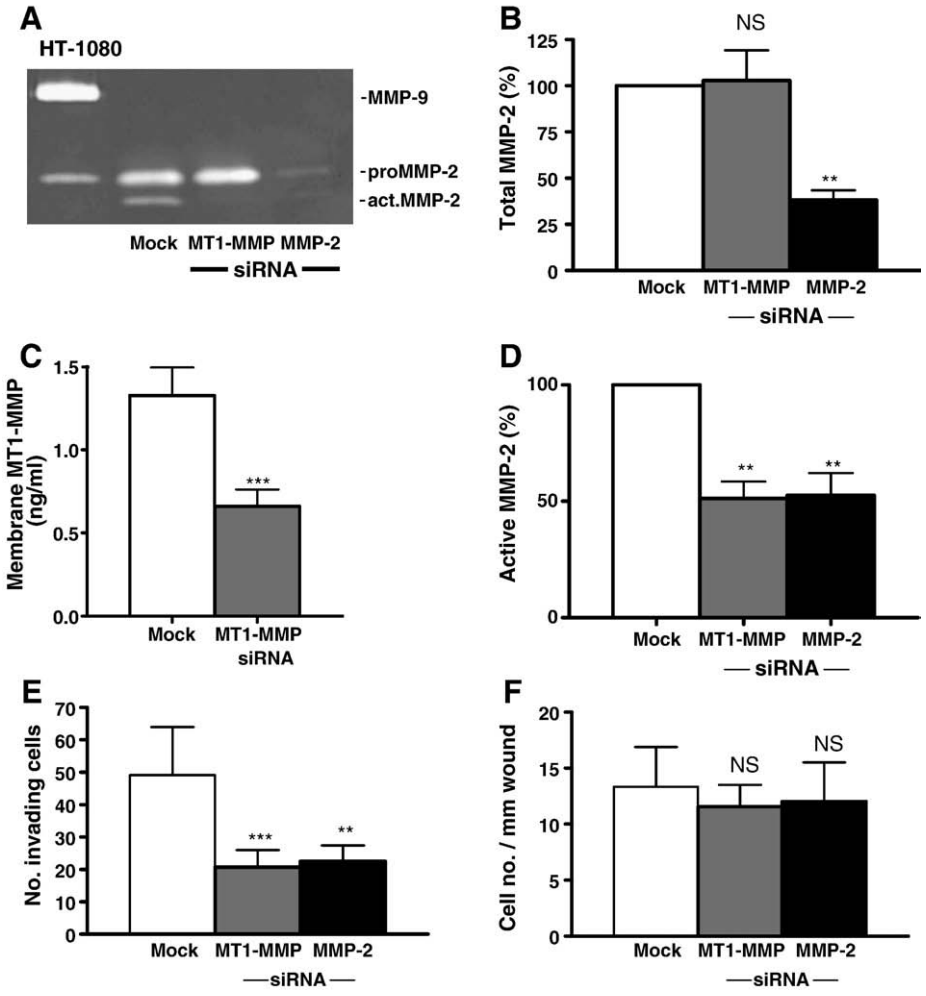

Fig. 5. Effect of MMP-2 and MT1-MMP gene silencing on MMP-2 secretion and activation, and myofibroblast function. For MMP quantification, mock-transfected and siRNA-transfected cardiac myofibroblasts were cultured in collagen for 48 h. Functional assays were performed in parallel using mock-transfected and siRNA-transfected cardiac myofibroblasts. (A) Representative zymography of conditioned media collected at 48 h. (B) Densitometric analysis of MMP-2, NS = not significant, $^{**}P<0.01$ ($n=5$). (C) Membrane MT1-MMP expression determined by ELISA. $^{***}P<0.01$ ($n=5$). (D) Densitometric quantification of active MMP-2 relative to mock-transfected cells. $^{**}P<0.01$ ($n=5$). (E) Quantification of invaded cells, $^{**}P<0.01$, $^{***}P<0.001$, ($n=6$). (F) Quantification of migrated cells. NS = not significant ($n=6$).

dependent. For example, MMP-2 knockout rats exhibit preserved cardiac function after MI suggesting that excessive MMP-2 activation is deleterious [45]. In contrast, diabetic mice display reduced cardiac expression of both MMP-2 and MT1-MMP and increased fibrosis suggesting that increased MMP-2 activation plays a positive role [47]. Also worthy of note is that the time course of MMP-2 activation post-MI is variable between species; reportedly within 3 h in sheep infarct zone [48], but not evident in the mouse until 4 days post-MI [49].

In conclusion, impaired cardiac myofibroblast invasion *in vivo* as a direct result of a period of oxygen deprivation may have deleterious sequelae on the myocardium by delaying early, adaptive remodelling following MI. Taking into account our own and previously published studies, it is clearly evident that species and cell-type variation is a major determinant of cellular responses to distinct stimuli. The multiplicity of reported differences between studies (for review see [50]) underscores the importance of selecting as far as possible, appropriate experimental models. Such considerations are of direct relevance to human disease, and our use of atrial-derived myofibroblasts may have imposed some constraint on the present study. Canine atrial and ventricular cells are claimed to exhibit inherent differences [51], but importantly, the behaviour of human atrial and ventricular cells has been reported to be similar [52]. The study described here, by the use of cardiac myofibroblasts from a series of patients in well established laboratory models of migration and invasion, endeavours to represent the *in vivo* scenario in man. Understanding the mechanisms of the effects of hypoxia on human cardiac myofibroblast function may ultimately reveal potential novel targets amenable to therapeutic manipulation.

5. Sources of funding

This work was supported in part by the British Heart Foundation (Junior Research Fellowship FS/05/067) awarded to MEM.

Acknowledgments

We are grateful to Stacey Galloway and Jean Kaye for cell culture expertise.

References

[1] Jugdutt BI. Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough? Circulation 2003;108:1395–403.
[2] Eghbali M. Cardiac fibroblasts: function, regulation of gene expression, and phenotypic modulation. Basic Res Cardiol 1992;87:183–9.
[3] Weber KT, Brilla CG, Janicki JS. Myocardial fibrosis: functional significance and regulatory factors. Cardiovasc Res 1993;27:341–8.
[4] Li PF, Dietz R, von Harsdorf R. Superoxide induces apoptosis in cardiomyocytes, but proliferation and expression of transforming growth factor-β1 in cardiac fibroblasts. FEBS Lett 1999;448:206–10.
[5] van Krimpen C, Smits JF, Cleutjens JP, Debets JJ, Schoemaker RG, Struyker Boudier HA, et al. DNA synthesis in the non-infarcted cardiac interstitium after left coronary artery ligation in the rat: effects of captopril. J Mol Cell Cardiol 1991;23:1245–53.
[6] Peterson JT, Hallak H, Johnson L, Li H, O Brien PM, Sliskovic DR, et al. Matrix metalloproteinase inhibition attenuates left ventricular remodeling and dysfunction in a rat model of progressive heart failure. Circulation 2001;103:2303–9.
[7] Rohde LE, Ducharme A, Arroyo LH, Aikawa M, Sukhova GH, Lopez-Anaya A, et al. Matrix metalloproteinase inhibition attenuates early left ventricular enlargement after experimental myocardial infarction in mice. Circulation 1999;99:3063–70.
[8] Seiki M, Yana I. Roles of pericellular proteolysis by membrane type-1 matrix metalloproteinase in cancer invasion and angiogenesis. Cancer Sci 2003;94:569–74.
[9] Yana I, Weiss SJ. Regulation of membrane type-1 matrix metalloproteinase activation by proprotein convertases. Mol Biol Cell 2000;11:2387–401.
[10] Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 1997;74:111–22.
[11] Seiki M, Koshikawa N, Yana I. Role of pericellular proteolysis by membrane-type 1 matrix metalloproteinase in cancer invasion and angiogenesis. Cancer Metastasis Rev 2003;22:129–43.
[12] Hu CP, Dandapat A, Liu Y, Hermonat PL, Mehta JL. Blockade of hypoxia-reoxygenation-mediated collagen type I expression and MMP activity by over-expression of TGF-beta1 delivered by AAV in mouse cardiomyocytes. Am J Physiol Heart Circ Physiol 2007;293:H1833–8.
[13] Strniskova M, Ravingerova T, Neckar J, Kolar F, Pastorekova S, Barancik M. Changes in the expression and/or activation of regulatory proteins in rat hearts adapted to chronic hypoxia. Gen Physiol Biophys 2006;25:25–41.
[14] Ottino P, Finley J, Rojo E, Ottlecz A, Lambrou GN, Bazan HE, et al. Hypoxia activates matrix metalloproteinase expression and the VEGF system in monkey choroid-retinal endothelial cells: involvement of cytosolic phospholipase A2 activity. Mol Vis 2004;10:341–50.
[15] Bergman MR, Cheng S, Honbo N, Piacentini L, Karliner JS, Lovett DH. A functional activating protein 1 (AP-1) site regulates matrix metalloproteinase 2 (MMP-2) transcription by cardiac cells through interactions with JunB-Fra1 and JunB-FosB heterodimers. Biochem J 2003;369:485–96.
[16] Fahling M, Perlewitz A, Doller A, Thiele BJ. Regulation of collagen prolyl 4-hydroxylase and matrix metalloproteinases in fibrosarcoma cells by hypoxia. Comp Biochem Physiol C Toxicol Pharmacol 2004;139:119–26.
[17] Ben-Yosef Y, Lahat N, Shapiro S, Bitterman H, Miller A. Regulation of endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation. Circ Res 2002;90:784–91.
[18] Zhang J, Salamonsen LA. Expression of hypoxia-inducible factors in human endometrium and suppression of matrix metalloproteinases under hypoxic conditions do not support a major role for hypoxia in regulating tissue breakdown at menstruation. Hum Reprod 2002;17:265–74.
[19] Orphanides C, Fine LG, Norman JT. Hypoxia stimulates proximal tubular cell matrix production via a TGF-beta1-independent mechanism. Kidney Int 1997;52:637–47.
[20] Turner NA, Porter KE, Smith WH, White HL, Ball SG, Balmforth AJ. Chronic β_2-adrenergic receptor stimulation increases proliferation of human cardiac fibroblasts via an autocrine mechanism. Cardiovasc Res 2003;57:784–92.
[21] Mughal RS, Warburton P, O Regan DJ, Ball SG, Turner NA, Porter KE. Peroxisome proliferator-activated receptor γ-independent effects of thiazolidinediones on human cardiac myofibroblast function. Clin Exp Pharmacol Physiol 2009;36:478–86.
[22] Porter KE, Turner NA, O Regan DJ, Balmforth AJ, Ball SG. Simvastatin reduces human atrial myofibroblast proliferation independently of cholesterol lowering via inhibition of RhoA. Cardiovasc Res 2004;61:745–55.
[23] Porter KE, Turner NA, O Regan DJ, Ball SG. Tumor necrosis factor α induces human atrial myofibroblast proliferation, invasion and MMP-9 secretion: inhibition by simvastatin. Cardiovasc Res 2004;64:507–15.
[24] Porter KE, Naik J, Turner NA, Dickinson T, Thompson MM, London NJ. Simvastatin inhibits human saphenous vein neointima formation via inhibition of smooth muscle cell proliferation and migration. J Vasc Surg 2002;36:150–7.
[25] Turner NA, Aley PK, Hall KT, Warburton P, Galloway S, Midgley L, et al. Simvastatin inhibits TNFα-induced invasion of human cardiac myofibroblasts via both MMP-9-dependent and -independent mechanisms. J Mol Cell Cardiol 2007;43:168–76.
[26] Spruill LS, Lowry AS, Stroud RE, Squires CE, Mains IM, Flack EC, et al. Membrane-type-1 matrix metalloproteinase transcription and translation in myocardial fibroblasts from patients with normal left ventricular function and from patients with cardiomyopathy. Am J Physiol Cell Physiol 2007;293:C1362–73.
[27] Gao Z, Sasaoka T, Fujimori T, Oya T, Ishii Y, Sabit H, et al. Deletion of the PDGFR-beta gene affects key fibroblast functions important for wound healing. J Biol Chem 2005;280:9375–89.
[28] Turner NA, Hall KT, Ball SG, Porter KE. Selective gene silencing of either MMP-2 or MMP-9 inhibits invasion of human saphenous vein smooth muscle cells. Atherosclerosis 2007;193:36–43.
[29] Guo C, Piacentini L. Type I collagen-induced MMP-2 activation coincides with up-regulation of membrane type 1-matrix metalloproteinase and TIMP-2 in cardiac fibroblasts. J Biol Chem 2003;278:46699–708.
[30] Maquoi E, Peyrollier K, Noel A, Foidart JM, Frankenne F. Regulation of membrane-type 1 matrix metalloproteinase activity by vacuolar H+-ATPases. Biochem J 2003;373:19–24.
[31] Ruangpanit N, Chan D, Holmbeck K, Birkedal-Hansen H, Polarek J, Yang C, et al. Gelatinase A (MMP-2) activation by skin fibroblasts: dependence on MT1-MMP expression and fibrillar collagen form. Matrix Biol 2001;20:193–203.
[32] Munoz-Najar UM, Neurath KM, Vumbaca F, Claffey KP. Hypoxia stimulates breast carcinoma cell invasion through MT1-MMP and MMP-2 activation. Oncogene 2006;25:2379–92.
[33] Cummins EP, Taylor CT. Hypoxia-responsive transcription factors. Pflugers Arch 2005;450:363–71.
[34] Lu KV, Jong KA, Rajasekaran AK, Cloughesy TF, Mischel PS. Upregulation of tissue inhibitor of metalloproteinases (TIMP)-2 promotes matrix metalloproteinase (MMP)-2 activation and cell invasion in a human glioblastoma cell line. Lab Invest 2004;84:8–20.
[35] Noda K, Ishida S, Shinoda H, Koto T, Aoki T, Tsubota K, et al. Hypoxia induces the expression of membrane-type 1 matrix metalloproteinase in retinal glial cells. Invest Ophthalmol Vis Sci 2005;46:3817–24.
[36] Deschamps AM, Yarbrough WM, Squires CE, Allen RA, McClister DM, Dowdy KB, et al. Trafficking of the membrane type-1 matrix metalloproteinase in ischemia and reperfusion: relation to interstitial membrane type-1 matrix metalloproteinase activity. Circulation 2005;111:1166–74.
[37] Fisher KE, Pop A, Koh W, Anthis NJ, Saunders WB, Davis GE. Tumor cell invasion of collagen matrices requires coordinate lipid agonist-induced G-protein and membrane-type matrix metalloproteinase-1-dependent signaling. Mol Cancer 2006;5:69.
[38] Ries C, Egea V, Karow M, Kolb H, Jochum M, Neth P. MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells: differential regulation by inflammatory cytokines. Blood 2007;109:4055–63.

[39] Sabeh F, Ota I, Holmbeck K, Birkedal-Hansen H, Soloway P, Balbin M, et al. Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. J Cell Biol 2004;167:769–81.
[40] Ueda J, Kajita M, Suenaga N, Fujii K, Seiki M. Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumors. Oncogene 2003;22:8716–22.
[41] Maquoi E, Noel A, Frankenne F, Angliker H, Murphy G, Foidart JM. Inhibition of matrix metalloproteinase 2 maturation and HT1080 invasiveness by a synthetic furin inhibitor. FEBS Lett 1998;424:262–6.
[42] Osenkowski P, Toth M, Fridman R. Processing, shedding, and endocytosis of membrane type 1-matrix metalloproteinase (MT1-MMP). J Cell Physiol 2004;200:2–10.
[43] Nyalendo C, Michaud M, Beaulieu E, Roghi C, Murphy G, Gingras D, et al. Src-dependent phosphorylation of membrane type I matrix metalloproteinase on cytoplasmic tyrosine 573: role in endothelial and tumor cell migration. J Biol Chem 2007;282:15690–9.
[44] Alfonso-Jaume MA, Bergman MR, Mahimkar R, Cheng S, Jin ZQ, Karliner JS, et al. Cardiac ischemia-reperfusion injury induces matrix metalloproteinase-2 expression through the AP-1 components FosB and JunB. Am J Physiol Heart Circ Physiol 2006;291:H1838–46.
[45] Schulz R, Heusch G. Tumor necrosis factor-alpha and its receptors 1 and 2: Yin and Yang in myocardial infarction? Circulation 2009;119:1355–7.
[46] Frantz S, Hu K, Adamek A, Wolf J, Sallam A, Maier SK, et al. Transforming growth factor beta inhibition increases mortality and left ventricular dilatation after myocardial infarction. Basic Res Cardiol 2008;103:485–92.
[47] Van LS, Seeland U, Riad A, Eckhardt O, Hohl M, Dhayat N, et al. Reduced MMP-2 activity contributes to cardiac fibrosis in experimental diabetic cardiomyopathy. Basic Res Cardiol 2008;103:319–27.
[48] Gallagher G, Menzie S, Huang Y, Jackson C, Hunyor SN. Regional cardiac dysfunction is associated with specific alterations in inflammatory cytokines and matrix metalloproteinases after acute myocardial infarction in sheep. Basic Res Cardiol 2007;102:63–72.
[49] Vanhoutte D, Schellings M, Pinto Y, Heymans S. Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: a temporal and spatial window. Cardiovasc Res 2006;69:604–13.
[50] Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol Ther 2009;123:255–78.
[51] Burstein B, Libby E, Calderone A, Nattel S. Differential behaviors of atrial versus ventricular fibroblasts: a potential role for platelet-derived growth factor in atrial-ventricular remodeling differences. Circulation 2008;117:1630–41.
[52] Hafizi S, Wharton J, Morgan K, Allen SP, Chester AH, Catravas JD, et al. Expression of functional angiotensin-converting enzyme and AT1 receptors in cultured human cardiac fibroblasts. Circulation 1998;98:2553–9.